IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC GLYCOSYLTRANSFERASE

Field of the Invention

The present invention relates to nucleic acids which encode glycosyltransferase and are useful in producing cells and organs from one species which may be used for transplantation into a recipient of another species. Specifically the invention concerns production of nucleic acids which, when present in cells of a transplanted organ, result in reduced levels of antibody recognition of the transplanted organ.

Background of the Invention

The transplantation of organs is now practicable, due to major advances in surgical and other techniques. However, availability of suitable human organs for transplantation is a significant problem. Demand outstrips supply. This has caused researchers to investigate the possibility of using non-human organs for transplantation.

Xenotransplantation is the transplantation of organs from one species to a recipient of a different species. Rejection of the transplant in such cases is a particular problem, especially where the donor species is more distantly related, such as donor organs from pigs and sheep to human recipients. Vascular organs present a special difficulty because of hyperacute rejection (HAR).

HAR occurs when the complement cascade in the recipient is initiated by binding of antibodies to donor endothelial cells.

Previous attempts to prevent HAR have focused on two strategies : modifying the immune system of the host by inhibition of systemic complement formation (1,2), and antibody depletion (3,4). Both strategies have been shown to prolong xenograft survival temporarily. However, these methodologies are therapeutically unattractive in that they are clinically impractical, and would require chronic

immunosuppressive treatments. Therefore, recent efforts to inhibit HAR have focused on genetically modifying the donor xenograft. One such strategy has been to achieve high-level expression of species-restricted human complement inhibitory proteins in vascularized pig organs via transgenic engineering (5-7). This strategy has proven to be useful in that it has resulted in the prolonged survival of porcine tissues following antibody and serum challenge (5,6). Although increased survival of the transgenic tissues was observed, long-term graft survival was not achieved (6). As observed in these experiments and also with systemic complement depletion, organ failure appears to be related to an acute antibody-dependent vasculitis (1,5).

In addition to strategies aimed at blocking complement activation on the vascular endothelial cell surface of the xenograft, recent attention has focused on identification of the predominant xenogeneic epitope recognised by high-titre human natural antibodies. It is now accepted that the terminal galactosyl residue, Gal-α(1,3)-Gal, is the dominant xenogeneic epitope (8-15). This epitope is absent in Old World primates and humans because the α(1,3)-galactosyltransferase (gal-transferase or GT) is non-functional in these species. DNA sequence comparison of the human gene to α(1,3)-galactosyltransferase genes from the mouse (16,17), ox (18), and pig (12) revealed that the human gene contained two frameshift mutations, resulting in a non-functional pseudogene (20,21). Consequently, humans and Old World primates have pre-existing high-titre antibodies directed at this Gal-α(1,3)-Gal moiety as the dominant xenogeneic epitope.

One strategy developed was effective to stably reduce the expression of the predominant Gal-α(1,3)-Gal epitope. This strategy took advantage of an intracellular competition between the gal-transferase and α(1,2)-fucosyltransferase (H-transferase) for a common acceptor substrate. The gal-transferase catalyzes the transfer of a

terminal galactose moiety to an N-acetyl lactosamine acceptor substrate, resulting in the formation of the terminal Gal-α(1,3)-Gal epitope. Conversely, H-transferase catalyzes ~~catalyses~~ the transfer of a fucosyl residue to the N-acetyl lactosamine acceptor substrate, and generates a fucosylated N-acetyl lactosamine (H-antigen, *i.e.*, the O blood group antigen), a glycosidic structure that is universally tolerated. Although it was reported that expression of human H-transferase transfected cells resulted in high level expression of the non-antigenic H-epitope and significantly reduced the expression of the Gal-α(1,3)-Gal xenoepitope, there are still significant levels of Gal-α(1,3)-Gal epitope present on such cells.

Summary of the Invention

In view of the foregoing, it is an object of the present invention to further reduce levels of undesirable epitopes in cells, tissues and organs which may be used in transplantation.

In work leading up to the invention the inventors surprisingly discovered that the activity of H transferase may be further increased by making a nucleic acid which encodes a H transferase catalytic domain but is anchored in the cell at a location where it is better able to compete for substrate with gal transferase. Although work by the inventors focused on a chimeric H transferase, other glycosyltransferase enzymes may also be produced in accordance with the invention.

Accordingly, in a first aspect the invention provides a nucleic acid encoding a chimeric enzyme, wherein said chimeric enzyme comprises a catalytic domain of a first glycosyltransferase and a localization ~~localisation~~ signal of a second glycosyltransferase, whereby when said nucleic acid is expressed in a cell said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with a second glycosyltransferase, resulting in reduced levels of a product from said second

glycosyltransferase.

Preferably the nucleic acid is in an isolated form; that is the nucleic acid is at least partly purified from other nucleic acids or proteins.

Preferably the nucleic acid comprises the correct sequences for expression, more preferably for expression in a eukaryotic cell. The nucleic acid may be present on any suitable eukaryotic expression vector such as pcDNA (Invitrogen). The nucleic acid may also be present on other vehicles whether suitable for eukaryotes or not, such as plasmids, phages and the like.

Preferably the catalytic domain of the first glycosyltransferase is derived from H transferase, secretor sialyltransferase, a galactosyl sulphating enzyme or a phosphorylating enzyme.

The nucleic acid sequence encoding the catalytic domain may be derived from, or similar to a glycosyltransferase from any species. Preferably said species is a mammalian species such as human or other primate species, including Old World monkeys, or other mammals such as ungulates (for example pigs, sheep, goats, cows, horses, deer, camels) or dogs, mice, rats and rabbits. The term "similar to" means that the nucleic acid is at least partly homologous to the glycocyltransferase genes described above. The term also extends to fragments of and mutants, variants and derivatives of the catalytic domain whether naturally occurring or man made.

Preferably the localisation signal is derived from a glycosyltransferase which produces glycosylation patterns which are recognised as foreign by a transplant recipient. More preferably the ~~localisation~~ localization signal is derived from α(1,3) galactosyltransferase. The effect of this is to downregulate the level of Gal-α(1,3)-Gal produced in a cell when the nucleic acid is expressed by the cell.

The nucleic acid sequence encoding the ~~localisation~~ localization signal may be derived from any species such as

those described above. Preferably it is derived from the same species as the cell which the nucleic acid is intended to transform i.e., if pig cells are to be transformed, preferably the localization signal is derived from pig.

More preferably the nucleic acid comprises a nucleic acid sequence encoding the catalytic domain of H transferase and a nucleic acid sequence encoding a localization signal from Gal transferase. Still more preferably both nucleic acid sequences are derived from pigs. Even more preferably the nucleic acid encodes gtHT described herein.

The term "nucleic acid" refers to any nucleic acid comprising natural or synthetic purines and pyrimidines. The nucleic acid may be DNA or RNA, single or double stranded or covalently closed circular.

The term "catalytic domain" of the chimeric enzyme refers to the amino acid sequences necessary for the enzyme to function catalytically. This comprises one or more contiguous or non-contiguous amino acid sequences. Other non-catalytically active portions also may be included in the chimeric enzyme.

The term "glycosyltransferase" refers to a polypeptide with an ability to move carbohydrates from one molecule to another.

The term "derived from" means that the catalytic domain is based on, or is similar, to that of a native enzyme. The nucleic acid sequence encoding the catalytic domain is not necessarily directly derived from the native gene. The nucleic acid sequence may be made by polymerase chain reaction (PCR), constructed *de novo* or cloned.

The term "localization signal" refers to the amino acid sequence of a glycosyltransferase which is responsible for anchoring it in location within the cell. Generally localization signals comprise amino terminal "tails" of the enzyme. The localization signals are derived from a second glycosyltransferase, the activity of which it is desired to minimise. The localization of a

catalytic domain of a first enzyme in the same area as the second glycosyltransferase means that the substrate reaching that area is likely to be acted on by the catalytic domain of the first enzyme, enabling the amount of substrate catalysed by the second enzyme to be reduced.

The term "area of the cell" refers to a region, compartment or organelle of the cell. Preferably the area of the cell is a secretory organelle such as the Golgi apparatus.

In another aspect the invention provides an isolated nucleic acid molecule encoding a localisation signal of a glycosyltransferase. Preferably the signal encoded comprises an amino terminus of said molecule; more preferably it is the amino terminus of gal transferase. The gal transferase may be derived from or based on a gal transferase from any mammalian species, such as those described above. Particularly preferred sequences are those derived from pig, mouse or cattle.

In another aspect the invention relates to a method of producing a nucleic acid encoding a chimeric enzyme, said enzyme comprising a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase whereby when said nucleic acid is expressed in a cell said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with a second glycosyltransferase said method comprising operably linking a nucleic acid sequence encoding a catalytic domain from a first glycosyltransferase to a nucleic acid sequence encoding a localisation signal of a second glycosyltransferase.

The term "operably linking" means that the nucleic acid sequences are ligated such that a functional protein is able to be transcribed and translated.

Those skilled in the art will be aware of various techniques for producing the nucleic acid. Standard techniques such as those described in Sambrook et al may be employed.

Preferably the nucleic acid sequences are the preferred sequences described above.

In another aspect the invention provides a method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localization signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

The term "reducing the level of a carbohydrate" refers to lowering, minimising, or in some cases, ablating the amount of carbohydrate displayed on the surface of the cell. Preferably said carbohydrate is capable of stimulating recognition of the cell as "non-self" by the immune system of an animal. The reduction of such a carbohydrate therefore renders the cell, or an organ composed of said cells, more acceptable to the immune system of a recipient animal in a transplant situation or gene therapy situation.

The term "causing a nucleic acid to be expressed" means that the nucleic acid is introduced into the cell (i.e. by transformation/transfection or other suitable means) and contains appropriate signals to allow expression in the cells.

The cell may be any suitable cell, preferably mammalian, such as that of a New World monkey, ungulate (pig, sheep, goat, cow, horse, deer, camel, etc.) or other species such as dogs.

In another aspect the invention provides a method of producing a cell from one species (the donor) which is immunologically acceptable to another species (the recipient) by reducing levels of carbohydrate on said cell

which cause it to be recognised as non-self by the other species, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a ~~localisation~~ localization signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

The term "immunologically acceptable" refers to producing a cell, or an organ made up of numbers of the cell, which does not cause the same degree of immunological reaction in the recipient species as a native cell from the donor species. Thus the cell may cause a lessened immunological reaction, only requiring low levels of immunosuppressive therapy to maintain such a transplanted organ or no immunosuppression therapy.

The cell may be from any of the species mentioned above. Preferably the cell is from a New World primate or a pig. More preferably the cell is from a pig.

The invention extends to cells produced by the above method and also to organs comprising the cells.

The invention further extends to non-human transgenic animals harbouring the nucleic acid of the invention. Preferably the species is a human, ape or Old World monkey.

The invention also extends to the proteins produced by the nucleic acid. Preferably the proteins are in an isolated form.

In another aspect the invention provides an expression unit which expresses the nucleic acid of the invention, resulting in a cell which is immunologically acceptable to an animal having reduced levels of a carbohydrate on its surface, which carbohydrate is ~~recognised~~ recognized as non-self by said species. In a preferred embodiment, the expression unit is a retroviral packaging

cell, cassette, a retroviral construct or retroviral producer cell.

Preferably the species is a human, ape or Old World monkey.

The retroviral packaging cells or retroviral producer cells may be cells of any animal origin where it is desired to reduce the level of carbohydrates on its surface to make it more immunologically acceptable to a host. Such cells may be derived from mammals such as canine, rodent or ruminant species and the like.

The retroviral packaging and/or producer cells may be used in applications such as gene therapy. General methods involving use of such cells are described in PCT/US95/07554 and the references discussed therein.

The invention also extends to a method of producing a retroviral packaging cell or a retroviral producer cell having reduced levels of a carbohydrate on its surface wherein the carbohydrate is recognised as non-self by a species, comprising transforming/transfecting a retroviral packaging cell or a retroviral producer cell with the nucleic acid of the invention under conditions such that the chimeric enzyme is produced.

Brief Description of the Drawings

Figure 1 **Schematic diagram of normal and chimeric glycosyltransferases**

The diagram shows normal glycosyltransferases porcine α(1,3)galactosyltransferase (GT) and human α(1,2)fucosyltransferase (HT), and chimeric transferases ht-GT in which the cytoplasmic domain of GT has been completely replaced by the cytoplasmic domain of HT, and gt-HT in which the cytoplasmic domain of HT has been entirely replaced by the cytoplasmic domain of GT. The protein domains depicted are cytoplasmic domain CYTO, transmembrane domain TM, stem region STEM, catalytic domain CATALYTIC. The numbers refer to the amino acid sequence of

the corresponding normal transferase.

Figure 2 Cell surface staining of COS cells transfected with normal and chimeric transferases

Cells were transfected with normal GT or HT or with chimeric transferases gt-HT or ht-GT and 48h later were stained with FITC-labelled lectin IB4 or UEAI. Positive-staining cells were visualised and counted by fluorescence microscopy. Results are from at least three replicates and values are +/- SEM.

Figure 3. RNA analysis of transfected COS cells

Northern blots were performed on total RNA prepared from COS cells transfected: Mock, mock-transfected; GT, transfected with wild-type GT; GT1-6/HT, transfected with chimeric transferase gt-HT; GT1-6/HT + HT1-8/GT, co-transfected with both chimeric transferases gt-HT and ht-GT; HT1-8/GT, transfected with chimeric transferase ht-GT; HT, transfected with normal HT; GT + HT, co-transfected with both normal transferases GT and HT. Blots were probed with a cDNA encoding GT (Top panel), HT (Middle panel) or g-actin (Bottom panel).

Figure 4. Enzyme kinetics of normal and chimeric glycosyltransferases

Lineweaver-Burk plots for α(1,3) galactosyltransferase (□) and α(1,2)fucosyltransferase (■) to determine the apparent Km values for N-acetyl lactosamine. Experiments were performed in triplicate, plots shown are of mean values of enzyme activity of wild-type transferases, GT and HT, and chimeric proteins ht-GT and gt-HT in transfected COS cell extracts using phenyl-B-D Gal and N-acetyl lactosamine as acceptor substrates.

Figure 5. Staining of cells co-transfected with chimeric transferases

Cells were co-transfected with cDNAs encoding

normal transferases GT + HT (panels A, B), with chimeric transferases gt-HT + ht-GT (panels C, D), with HT + ht-GT (panels E, F) or with GT + gt-HT (panels G, H) and 48h later were stained with FITC-labelled lectin IB4 (panels A, C, E, G) or UEAI (panels B, D, F, H).

Figure 6 (SEQ ID NO:1) is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig secretor.

Figure 7 (SEQ ID NO:3) is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig H.

Figure 8 Cell surface staining of pig endothelial cell line (PIEC) transfected with chimeric α(1,2)-fucosyltransferase. Cells were transfected and clones exhibiting stable integration were stained with UEAI lectin and visualised by fluorescence microscopy.

Figure 9 Screening of chimeric α(1,2)-fucosyltransferase transferase in mice. Mice were injected with chimeric α(1,2)-fucosyltransferase and the presence of the transferase was analysed by dot blots.

Description of the Preferred Embodiment

The nucleic acid sequences encoding the catalytic domain of a glycosyltransferase may be any nucleic acid sequence such as those described in PCT/US95/07554, which is herein incorporated by reference, provided that it encodes a functional catalytic domain with the desired glycosyltransferase activity.

Preferred catalytic domains from glycosyltransferase include H transferase and secretor. Preferably these are based on human or porcine sequences.

The nucleic acid sequences encoding the localization ~~localisation~~ signal of a second transglycosylase may be any nucleic acid sequence encoding a signal sequence such as signal sequences disclosed in P A Gleeson, R D Teasdale &

J Bourke, Targeting of proteins to the Golgi apparatus. Glycoconjugate J. (1994) 11: 381-394. Preferably the localisation signal is specific for the Golgi apparatus, more preferably for that of the trans Golgi. Still more preferably the localization signal is based on that of Gal transferase. Even more preferably the localisation signal is based on porcine, murine or bovine sequences. Even more preferably the nucleic acid encodes a signal sequence with following amino acid sequence (in single letter code): MNVKGR (porcine) (SEQ. ID. NO. 11), MNVKGK (mouse) (SEQ. ID. NO. 12) or MVVKGK (bovine) (SEQ. ID. NO. 13).

Vectors for expression of the chimeric enzyme may be any suitable vector, including those disclosed in PCT/US95/07554.

The nucleic acid of the invention can be used to produce cells and organs with the desired glycosylation pattern by standard techniques, such as those disclosed in PCT/US95/07554. For example, embryos may be transfected by standard techniques such as microinjection of the nucleic acid in a linear form into the embryo (22). The embryos are then used to produce live animals, the organs of which may be subsequently used as donor organs for implantation.

Cells, tissues and organs suitable for use in the invention will generally be mammalian cells. Examples of suitable cells and tissues such as endothelial cells, hepatic cells, pancreatic cells and the like are provided in PCT/US95/07554.

The invention will now be described with reference to the following non-limiting Examples.

ABBREVIATIONS

The abbreviations used are bp, base pair(s); FITC, fluorescein isothiocyanate; GT, galactosyltransferase; H substance, α(1,2)fucosyl lactosamine; HT, α(1,2)fucosyltransferase; PCR, polymerase chain reaction;

Example 1 Cytoplasmic domains of glycosyltransferases play a central role in the temporal action of enzymes

EXPERIMENTAL PROCEDURES

Example 1 Plasmids - The plasmids used were prepared using standard techniques (7); pGT encodes the cDNA for the porcine α(1,3)galactosyltransferase (23), pHT encodes the cDNA for the α(1,2)fucosyltransferase (human) (25). Chimeric glycosyltransferase cDNAs were generated by polymerase chain reaction as follows: an 1105 bp product ht-GT was generated using primers corresponding to the 5′ end of ht-GT (5′-GCGGATCCATGTGGCTCCGGAGCC ATCGTCAGGTGGTTCTGTCAATGC TGCTTG-3′) (SEQ ID NO.5) coding for nucleotides 1-24 of HT (25) followed immediately by nucleotides 68-89 of GT (8) and containing a BamH1 site (underlined) and a primer corresponding to the 3′ end of ht-GT (5′-GCTCTAGAGCGTCAGATGTTATT TCTAACCAAATTATAC-3′) (SEQ ID NO.6) containing complementarity to nucleotides 1102-1127 of GT with an Xba1 site downstream of the translational stop site (underlined); an 1110 bp product gt-HT was generated using primers corresponding to the 5′ end of gt-HT (5′-GCGGATCCATGAATGTCAAAGGAAGACTCTGCCTGGCCT TCCTGC-3′) (SEQ ID NO.7) coding for nucleotides 49-67 of GT followed immediately by nucleotides 25-43 of HT and containing a BamH1 site (underlined) and a primer corresponding to the 3′ end of gt-HT (5′-GCTCTAGAGCCTCAAGGCTTAG CCAATGTCCAGAG-3′) (SEQ ID NO.8) containing complementarity to nucleotides 1075-1099 of HT with a Xba1 site downstream of the translational stop site (underlined). PCR products were restricted BamH1/Xba1, gel-purified and ligated into a BamH1/Xba1 digested pcDNA1

expression vector (Invitrogen) and resulted in two plasmids pht-GT (encoding the chimeric glycosyltransferase ht-GT) and pgt-HT (encoding the chimeric glycosyltransferase gt-HT) which were characterized by restriction mapping, Southern blotting and DNA sequencing .

Transfection and Serology - COS cells were maintained in Dulbecco's modified Eagles Medium (DMEM) (Trace Biosciences Pty. Ltd. , Castle Hill, NSW, Australia) and were transfected (1-10 µg DNA/5 x 105 cells) using DEAE-Dextran (26); 48h later cells were examined for cell surface expression of H substance or Gal-α(1,3)-Gal using FITC-conjugated lectins: IB4 lectin isolated from Griffonia simplicifolia (Sigma, St. Louis, MO) detects Gal-α(1,3)-Gal (27); UEAI lectin isolated from Ulex europaeus (Sigma, St. Louis, MO) detects H substance (28). H substance was also detected by indirect immunofluorescence using a monoclonal antibody (mAb) specific for the H substance (ASH-1952) developed at the Austin Research Institute, using FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) to detect mAb binding. Fluorescence was detected by microscopy.

RNA Analyses - Cytoplasmic RNA was prepared from transfected COS cells using RNAzol (Biotecx Laboratories, Houston, TX), and total RNA was electrophoresed in a 1% agarose gel containing formaldehyde, the gel blotted onto a nylon membrane and probed with random primed GT or HT cDNA.

Glycosyltransferase assays - Forty-eight hours after transfection, cells were washed twice with phosphate buffered saline and lysed in 1% Triton X-100/ 100 mM cacodylate pH 6. 5/ 25 mM MnCl2, at 4°C for 30 min; lysates were centrifuged and the supernatant collected and stored at -70°C. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (29). Assays for HT activity (30) were performed in 25 µl containing 3mM [GDP-^{14}C]fucose (specific activity 287 mCi/mmol, Amersham International), 5mM ATP, 50mM MOPS pH 6. 5, 20 mM MnCl2, using 2-10 µl of cell extract

(approximately 15-20µg of protein) and a range of concentrations (7. 5 -75 mM) of the acceptor phenyl-B-D-galactoside (Sigma). Samples were incubated for 2h at 37°C and reactions terminated by the addition of ethanol and water. The amount of ^{14}C-fucose incorporated was counted after separation from unincorporated label using Sep-Pak C18 cartridges (Waters-Millipore, Millford, MA). GT assays (31) were performed in a volume of 25 µl using 3mM UDP[^{3}H]-Gal (specific activity 189mCi/mmol, Amersham International), 5mM ATP, 100mM cacodylate pH 6. 5, 20mM $MnCl_2$ and various concentrations (1 -10 mM) of the acceptor N-acetyl lactosamine (Sigma). Samples were incubated for 2h at 37°C and the reactions terminated by the addition of ethanol and water. ^{3}H-Gal incorporation was counted after separation from non-incorporated UDP[^{3}H]-Gal using Dowex I anion exchange columns (BDH Ltd. , Poole, UK) or Sep-Pak Accell plus QMA anion exchange cartridges (Waters-Millipore, Millford, MA). All assays were performed in duplicate and additional reactions were performed in the absence of added acceptor molecules, to allow for the calculation of specific incorporation of radioactivity.

RESULTS

Expression of chimeric α(1,3) ~~galactosyltransferase~~ galactosyltransferase and α(1,2) ~~fucosyltransferase~~ fucosyltransferase cDNAs

We had previously shown that when cDNAs encoding α(1,3) ~~galactosyltransferase~~ galactosyltransferase (GT) and α(1,2)fucosyltransferase (HT) were transfected separately they could both function efficiently leading to expression of the appropriate carbohydrates: Gal-α(1,3)-Gal for GT and H substance for HT (32). However when the cDNAs for GT and HT were transfected together, the HT appeared to "dominate" over the GT in that H substance expression was normal, but Gal-α(1,3)-Gal was reduced. We excluded trivial reasons for this effect and considered that the localisation of the enzymes may be the reason. Thus, if the HT ~~localisation~~ localization signal placed the enzyme in an earlier temporal compartment

than GT, it would have "first use" of the N-acetyl lactosamine substrate. However, such a "first use" if it occurred, was not sufficient to adequately reduce GT. Two chimeric glycosyltransferases were constructed using PCR wherein the cytoplasmic tails of GT and HT were switched. The two chimeras constructed are shown in Fig.1: ht-GT which consisted of the NH_2 terminal cytoplasmic tail of HT attached to the transmembrane, stem and catalytic domains of GT; and gt-HT which consisted of the NH_2 terminal cytoplasmic tail of GT attached to the transmembrane, stem and catalytic domains of HT. The chimeric cDNAs were subcloned into the eukaryotic expression vector pcDNAI and used in transfection experiments.

The chimeric cDNAs encoding ht-GT and gt-HT were initially evaluated for their ability to induce glycosyltransferase expression in COS cells, as measured by the surface expression of the appropriate sugar using lectins. Forty-eight hours after transfection COS cells were tested by immunofluorescence for their expression of Gal-α(1,3)-Gal or H substance (Table 1 & Fig. 2). The staining with IB4 (lectin specific for Gal-α(1,3)-Gal) in cells expressing the chimera ht-GT (30% of cells stained positive) was indistinguishable from that of the normal GT staining (30%) (Table 1 & Fig. 2). Similarly the intense cell surface fluorescence seen with UEAI staining (the lectin specific for H substance) in cells expressing gt-HT (50%) was similar to that seen in cells expressing wild-type pHT (50%) (Table 1 & Fig. 2). Furthermore, similar levels of mRNA expression of the glycosyltransferases GT and HT and chimeric glycosyltransferases ht-GT and gt-HT were seen in Northern blots of total RNA isolated from transfected cells (Fig. 3). Thus both chimeric glycosyltransferases are efficiently expressed in COS cells and are functional indeed there was no detectable difference between the chimeric and normal glycosyltransferases.

Glycosyltransferase activity in cells transfected with chimeric cDNAs encoding ht-GT and gt-HT

To determine whether switching the cytoplasmic tails of GT and HT altered the kinetics of enzyme function, we compared the enzymatic activity of the chimeric glycosyltransferases with those of the normal enzymes in COS cells after transfection of the relevant cDNAs. By making extracts from transfected COS cells and performing GT or HT enzyme assays we found that N-acetyl lactosamine was galactosylated by both GT and the chimeric enzyme ht-GT (Fig 4. panel A) over a the 1-5mM range of substrate concentrations. Lineweaver-Burk plots showed that both GT and ht-GT have a similar apparent Michealis-Menten constant of Km 2. 6mM for N-acetyl lactosamine (Fig. 4. panel B). Further HT, and the chimeric enzyme gt-HT were both able to fucosylate phenyl-B-D-galactoside over a range of concentrations (7. 5 - 25 mM) (Fig. 4 panel C) with a similar Km of 2. 3mM (Fig. 4 panel D), in agreement with the reported Km of 2. 4mM for HT (25). Therefore the chimeric glycosyltransferases ht-GT and gt-HT are able to utilize ~~utilise~~ N-acetyl lactosamine (ht-GT) and phenyl-B-D-galactoside (gt-HT) in the same way as the normal glycosyltransferases, thus switching the cytoplasmic domains of GT and HT does not alter the function of these glycosyltransferases and if indeed the cytoplasmic tail is the localization ~~localisation~~ signal then both enzymes function as well with the GT signal as with the HT signal.

Switching cytoplasmic domains of GT and HT results in a reversal of the "dominance" of the glycosyltransferases

The cDNAs encoding the chimeric transferases or normal transferases were simultaneously co-transfected into COS cells and after 48h the cells were stained with either IB4 or UEA1 lectin to detect Gal-α(1,3)-Gal and H substance respectively on the cell surface (Table 1 & Fig. 5). COS cells co-transfected with cDNAs for ht-GT + gt-HT (Fig 5 panel C) showed 30 % cells staining positive with IB4

(Table 1) but no staining on cells co-transfected with cDNAs for GT + HT (3%) (Fig. 5 panel A). Furthermore staining for H substance on the surface of ht-GT + gt-HT co-transfectants gave very few cells staining positive (5%) (Fig 5 panel D) compared to the staining seen in cells co-transfected with cDNAs for the normal transferases GT + HT (50%) (Fig. 5 panel B), ie. the expression of Gal-α(1,3)-Gal now dominates over that of H. Clearly, switching the cytoplasmic tails of GT and HT led to a complete reversal in the glycosylation pattern seen with the normal transferases i.e. the cytoplasmic tail sequences dictate the pattern of carbohydrate expression observed.

That exchanging the cytoplasmic tails of GT and HT reverses the dominance of the carbohydrate epitopes points to the glycosyltransferases being relocalized within the Golgi. To address this question, experiments were performed with cDNAs encoding glycosyltransferases with the same cytoplasmic tail: COS cells transfected with cDNAs encoding HT + ht-GT stained strongly with both UEAI (50%) and IB4 (30%) (Table 1 & Fig. 5 panels E, F), the difference in staining reflecting differences in transfection efficiency of the cDNAs. Similarly cells transfected with cDNAs encoding GT + gt-HT also stained positive with UEAI (50%) and IB4 (30%) (Table 1 & Fig. 5 panel G, H). Thus, glycosyltransferases with the same cytoplasmic tail leads to equal cell surface expression of the carbohydrate epitopes, with no "dominance" of one glycosyltransferase over the other observed, and presumably the glycosyltransferases ~~localised~~ localized at the same site appear to compete equally for the substrate.

In COS cells the levels of transcription of the cDNAs of chimeric and normal glycosyltransferases were essentially the same (Fig.3) and the immunofluorescence pattern of COS cells expressing the chimeric glycosyltransferases ht-GT and gt-HT showed the typical staining pattern of the cell surface Gal-α(1,3)-Gal and H substance respectively (Table 1 & Fig. 2), the pattern

being indistinguishable from that of COS cells expressing normal GT and HT. Our studies showed that the Km of ht-GT for N-acetyl lactosamine was identical to the Km of GT for this substrate, similarly the Km of gt-HT for phenylBDgalactoside was approximately the same as the Km of HT for phenylbDgalactoside (Fig. 3). These findings indicate that the chimeric enzymes are functioning in a cytoplasmic tail-independent manner, such that the catalytic domains are entirely functional, and are in agreement with those of Henion et al (23), who showed that an NH_2 terminal truncated marmoset GT (including truncation of the cytoplasmic and transmembrane domains) maintained catalytic activity and confirmed that GT activity is indeed independent of the cytoplasmic domain sequence.

If the Golgi localisation signal for GT and HT is contained entirely within the cytoplasmic domains of the enzymes, then switching the cytoplasmic tails between the two transferases should allow a reversal of the order of glycosylation. Co-transfection of COS cells with cDNA encoding the chimeric glycosyltransferases ht-GT and gt-HT caused a reversal of staining observed with the wild type glycosyltransferases (Fig. 5), demonstrating that the order of glycosylation has been altered by exchanging the cytoplasmic tails. Furthermore, co-transfection with cDNA encoding glycosyltransferases with the same cytoplasmic tails (i. e. HT + ht-GT and GT + gt-HT) gave rise to equal expression of both Gal-α(1,3)-Gal and H substance (Fig.5). The results imply that the cytoplasmic tails of GT and HT are sufficient for the localization ~~localisation~~ and retention of these two enzymes within the Golgi.

To date only twenty or so of at least one hundred predicted glycosyltransferases have been cloned and few of these have been studied with respect to their Golgi localization ~~localisation~~ and retention signals (34). Studies using the elongation transferase N-acetylglucosaminyltransferase I (33-37), the terminal transferases α(2,6)sialyltransferase (24-26) and β(1,4)galactosyltransferase (38-40) point to

residues contained within the cytoplasmic tail, transmembrane and flanking stem regions as being critical for Golgi localisation and retention. There are several examples of localization signals existing within cytoplasmic tail domains of proteins including the KDEL and KKXX motifs in proteins resident within the endoplasmic reticulum (41,42) the latter motif also having been identified in the cis Golgi resident protein ERGIC-53 (43) and a di-leucine containing peptide motif in the mannose-6-phosphate receptor which directs the receptor from the trans-Golgi network to endosomes (44). These motifs are not present within the cytoplasmic tail sequences of HT or GT or in any other reported glycosyltransferase. To date a localization signal in Golgi resident glycosyltransferases has not been identified and while there is consensus that transmembrane domains are important in Golgi localization, it is apparent that this domain is not essential for the localization of all glycosyltransferases, as shown by the study of Munro (45) where replacement of the transmembrane domain of α(2,6)sialyltransferase in a hybrid protein with a poly-leucine tract resulted in normal Golgi retention. Dahdal and Colley (46) also showed that sequences in the transmembrane domain were not essential to Golgi retention. This study is the first to identify sequence requirements for the localisation of α(1,2)fucosyltransferase and α(1,3)galactosyltransferase within the Golgi. It is anticipated that other glycosyltransferases will have similar localization mechanisms.

Example 2 Use of secretor in construction of a chimeric enzyme

A construct is made using PCR and subcloning as described in Example 1, such that amino acids #1 to #6 of the pig α(1,3)-galactosyltransferase (MNVKGR) replace amino acids #1 to 5 of the pig secretor (Fig 6). Constructs are tested as described in Example 1.

Example 3 Use of pig H transferase in construction of a chimeric enzyme

A construct is made using PCR and subcloning as described in Example 1, such that amino acids #1 to #6 of the pig α(1,3)-galactosyltransferase (MNVKGR) (SEQ. ID NO 14), replace amino acids #1 to 8 of the pig H transferase (Fig 7). Constructs are tested as described in Example 1.

Example 4. Generation of pig endothelial cells expressing chimeric α(1,2)fucosyltransferase

The pig endothelial cell line PIEC expressing the chimeric α1,2fucosyltransferase was produced by lipofectamine transfection of pgtHT plasmid DNA (20 μg) and pSV2NEO (2 μg) and selecting for stable integration by growing the transfected PIEC in media containing G418 (500 μg/ml; Gibco-BRL, Gaithersburg, MD). Fourteen independant clones were examined for cell surface expression of H substance by staining with UEA-1 lectin. >95% of cells of each of these clones were found to be positive. Fig. 8 shows a typical FACS profile obtained for these clones.

Example 5 Production of transgenic mice expressing chimeric α(1,2)fucosyltransferase

A NruI/NotI DNA fragment, encoding the full length chimeric α1,2fucosyltransferase, was generated utilizing the Polymerase Chain Reaction and the phHT plasmid using the primers:

5' primer homologous to the 5'UTR:

5'-TTCGCGAATGAATGTCAAAGGAAGACTCTG, (SEQ ID NO.9) in which the underlined sequence contains a unique NruI site;

3' primer homologous to the 3'UTR:

5'-GGCGGCCGCTCAGATGTTATTTCTAACCAAAT (SEQ ID NO 10)

the underlined sequence contains a NotI site

The DNA was purified on gels, electroeluted and subcloned into a NruI/NotI cut genomic H-2Kb containing vector resulting in the plasmid clone (pH-2Kb-gtHT)

encoding the chimeric α(1,2)-fucosyltransferase gene directionally cloned into exon 1 of the murine H-2Kb gene, resulting in a transcript that commences at the H-2Kb transcriptional start site, continuing through the gtHT cDNA insert. The construct was engineered such that translation would begin at the initiation codon (ATG) of the hHT cDNA and terminate at the in-phase stop codon (TGA).

DNA was prepared for microinjection by digesting pH-2Kb-hHT with XhoI and purification of the H-2Kb-hHT DNA from vector by electrophoretic separation in agarose gels, followed by extraction with chloroform, and precipitation in ethanol to decontaminate the DNA. Injections were performed into the pronuclear membrane of (C57BL/6xSJL)F1 zygotes at concentrations between 2-5ng/ml, and the zygotes transferred to pseudopregnant (C57BL/6xSJL)F1 females.

The presence of the transgene in the live offspring was detected by dot blotting. 5mg of genomic DNA was transferred to nylon filters and hybridized with the insert from gtHT, using a final wash at 68°C in 0.1xSSC/1% SDS. Fig. 9 shows the results of testing 12 live offspring, with two mice having the transgenic construct integrated into the genome. Expression of transgenic protein is examined by estimating the amount of UEAI lectin (specific for H substance) or anti-H mAb required to haemagglutinate red blood cells from transgenic mice. Hemagglutination in this assay demonstrates transgene expression.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the following pages, and are incorporated herein by this

reference.

TABLE 1

EXPRESSION OF GAL-α(1,3)GAL AND H SUBSTANCE BY COS CELLS TRANSFECTED WITH cDNAs ENCODING NORMAL AND CHIMERIC GLYCOSYLTRANSFERASES

COS cells transfected with cDNA encoding:	%IB4 positive cells	%UEAI positive cells
GT	30	0
HT	0	50
ht-GT	30	0
gt-HT	3	50
GT+HT	3	50
ht-GT+gt-HT	33	5
GT+gt-HT	30	30
GT+ht-GT	30	0
HT+ht-GT	30	30
HT+gt-HT	0	50
Mock	0	0

Transfected COS cells were stained with FITC-labelled IB4 (lectin specific for Gal-α(1,3)Gal or UEAI (lectin specific for H substance) and positive staining cells were visualized and counted by fluorescence microscopy. Results are from at least three replicates.

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